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Cytokine Profiles in Obese African Americans following Influenza Vaccination

By

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Honors Thesis

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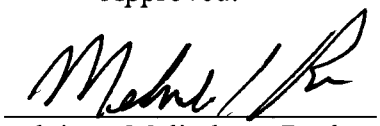
Approved:



Advisor: Melinda A. Beck

Reader:

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ABSTRACT

Obesity is one of modern day's most serious public health concerns, costing billions of dollars and claiming millions of lives every year. In addition to being a co-morbidity for significant health concerns and diseases, obesity has been proven to have significant negative effects on the effectiveness of vaccination for the influenza virus. The influenza virus is responsible for killing more than 5% of those who contract it. This study looks at IL-6 and CRP, inflammation cytokines, to look at the whether increased serum levels of IL-6 and CRP in subjects with higher BMI values result in reduced responses to vaccination. Results showed that there was no significant correlation between IL-6 levels and CRP levels and response to vaccination. Obese subjects did have higher levels of these cytokines than literature values for healthy weight individuals. Older individuals and high responders to the vaccination were both linked to lower levels of IL-6 within the serum. Although no significant results were found, this study should be performed again with a larger and more diverse sample size that includes healthy weight subjects to better understand the links between serum cytokine levels and response to vaccination.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1 Obesity

Obesity has quickly become one of America's most predominant public health concerns. More than one third, or a total of 78.6 million U.S. adults are obese while 2 out of three adults are considered to be overweight or obese. Since the early 1960s, the prevalence of obesity among adults more than doubled, increasing from 13.4 to 35.7 percent in U.S. adults age 20 and older.[1] Obesity is a multifaceted disorder associated with an excessive amount of fat dispersed throughout the body. Excess body fat increases the likelihood of health problems and diseases such heart disease, stroke, high blood pressure, certain types of cancer, and type 2 diabetes. Furthermore, the obesity epidemic costs the United States more than $148 billion a year, with medical costs for obese individuals being $1,429 higher than those of healthy weights. In addition to being a cosmetic and physical concern, obesity may also result in social and psychological concerns.

Excess weight gain leading up to overweightness and then obesity can be attributed to a multitude of genetic, environmental, and lifestyle factors. The most common causes of obesity are overeating and physical inactivity where positive energy imbalance exists. Genetic factors can influence obesity and can enhance the effects of an environment and lifestyle that promotes weight gain. Environmental and social factors such as having limited access to nutritious food and adequate resources for physical activity can further result in obesity. Additional factors that contribute to obesity include the use of certain medications, medical syndromes that limit activity, lack of sleep, and pregnancy.

Obesity can be determined through skinfold tests, waist circumference measures, bioelectric impedance, underwater weighing, or most commonly through the calculation of an individual's body mass index (BMI).[3] An individual's BMI is calculated by dividing one's weight in kilograms by their height squared in meters. According to the Centers for Disease Control and Prevention (CDC), an adult with a BMI lower than 18.5 is classified as underweight, an adult with a BMI between 18.5 and 24.9 is classified as healthy weight, an adult with a BMI between 25.0 and 29.9 is classified as overweight, and an adult with a BMI 30.0 and above is classified as obese. The most obvious limitation to this practice is that BMI makes no distinction between the weight of adipose fat and the weight of muscle. Individuals with developed muscles commonly have much higher BMIs and may be incorrectly diagnosed as overweight or obese.

1.2 Influenza Virus

Influenza is one of the most commonly spread infectious viruses, affecting between 5 and 20% of affected Americans every year.[8] Influenza is a serious illness that can lead to hospitalization or even death. Symptoms of influenza include fever, runny nose, coughing, sore throat, muscle aches, fatigue, and headaches. Flu virus transmission or "flu season" most commonly occurs in the fall and winter seasons. Populations especially susceptible to influenza include the elderly, children, pregnant women, and those with weakened immune systems. Modes of transmission include sneezing, coughing, or through talking that forms droplets.[9] There are three different types of influenza virus: influenza A, influenza B, and influenza C. Influenza type A most commonly causes pandemics and is most threatening to humans, birds, and pigs. Influenza type B infects humans, seals, and ferrets, and influenza type C infects both

humans and some animals. Influenza A is composed of 8 RNA segments and is classified into subtypes that depend on which hemmagglutinin (HA) protein and neuraminidase (NA) protein rests on the viral surface. Furthermore, there are 18 hemagglutinin subtypes and 11 neuraminidase subtypes.[10] These hemmagglutinin and neuraminidase subtypes make up the name of each flu virus. The letters and numbers stand for specific components of the virus that set it apart from others.[14] In the case of H3N2, the virus has hemagglutinin type 3 (H3) and neuraminidase type 2 (N2), constituents that occur on the surface of the virus.

The influenza virus is especially potent because it changes so rapidly through antigenic drift and antigenic shift.[11] Antigenic drift is a mechanism for variation by viruses that involves the accumulation of mutations in the HA and NA viral genes that alter the antibody-binding sites so that the mutated viruses escape from antibodies generated from the unmutated virus. Although the changes may be miniscule, the new virus may not be recognizable by immune cells, making rapid transmission much more likely. Antigenic drift occurs in both influenza type A and influenza type B viruses. Antigeneic shift is a much more exceptional phenomenon and occurs when a new viral strain forms from two different HA and NA proteins to produce a new strain.[11]

1.3 Influenza Vaccine

The influenza vaccine attempts to prevent viral infection and spread of the illness by providing active acquired immunity. A vaccine contains agents that resemble disease-causing microorganisms made from weakened forms of the microbe or surface protein.[12] Exposure to this agent stimulates the body's immune system to recognize the threat, demolish it, and build antibodies to recognize it for a later encounter. This allows the

immune system to quickly respond and destroy the virus or agent when it comes into contact.

Everyone 6 months of age or older should get the yearly flu vaccine as early in the flu season as possible. The flu vaccination is usually offered by September but is continually available through the end of the flu season.[8] Since it takes almost two weeks after vaccination for antibodies to develop in the body that protect against the influenza virus infection, it is recommended that people get vaccinated as early as possible so that they are protected before influenza begins spreading in their community.[11] A flu vaccine is needed on an annual basis because the body's immune response from vaccination declines over time and because flu virus changes every year, meaning a previous year's vaccine will not suffice. Flu vaccines are manufactured in advance based on predictions of the upcoming season's strain. For the 2010-2011 year, the seasonal trivalent flu virus was composed of two subtypes of type A influenza and one subtype of type B influenza.[12] There is still a possibility of getting the flu even after getting vaccinated. Factors that affect the ability of the flu vaccine to protect a person include the health status and age of the person being vaccinated. Additionally, the vaccine can only be effective if the viruses used to make the vaccine match those circulating.[8]

After vaccination, the immune system responds to the antigens in the vaccine to destroy the virus. This stimulates memory B and T cells to recognize the antigen and quickly respond to the influenza strain upon later contact. Antigen-specific B lymphocytes that were induced during the original exposure remain in the body so that cells can quickly remove the infection once it returns.[9]

1.4 Obesity and Influenza Vaccination

Obesity has been determined to be associated with impaired immune response to influenza vaccination.[13] An ongoing prospective observational study at the University of North Carolina studied any potential links between a higher BMI and immune response to the influenza vaccine in human samples. Data from the first two years of the study revealed that obese and healthy weight individuals increased their IgG antibodies specific for the vaccine one month after vaccination. Conversely, the obese population had a greater decline in antibody titers than the healthy weight population one-year post-vaccination[13]. This decline in antibody titers in the obese population demonstrates that the obesity may have a diminished the immune response to the influenza vaccine. Obese individuals showed decreased CD8+ T cell activation and a lower amount of functional proteins in comparison to healthy weight individuals. Additionally, diet-induced obese mice have greater mortality rates to influenza infection and decreased innate immune response in comparison to healthy weight mice.[13]

1.5 C-Reactive Protein

C-Reactive Protein (CRP) is a critical component of the immune system produced by the liver and released into the blood after infection, tissue injury, or another source of inflammation. CRP is a complex set of proteins that was discovered in 1930 as a native protein and member of the small pentraxins family[5]. CRP functions by binding to the phosphocholine expressed on the surface of dead cells and then activating a complement system that promotes phagocytosis by macrophages. Acute-phase proteins, such as CRP, are proteins that increase or decrease in response to inflammation. This acute-phase response occurs as a result of increased interleukin- 6 (IL-6), which is produced by

macrophages and adipocytes. Infections, tissue injury, and inflammatory diseases cause the release of IL-6 among other cytokines that simulate the production of CRP by the liver. Serum CRP levels rise within two hours of inflammation by up to a 50,000-fold amount.[4] With a consistent half-life of 48 hours, CRP levels are influenced by the rate of production and therefore often used a screening for inflammation.

Genetic and lifestyle factors affect the amount of CRP an individual creates. Those who smoke, fail to exercise adequately, or have high blood pressure tend to have high levels of CRP.[4] Lean and athletic individuals of healthy weight tend to have lower levels of CRP in comparison. Genetic factors play an overbearing role in the variation of CRP levels as serum levels are inherited from parents and grandparents to children. Increased levels of CRP result in increased levels of inflammation, a vital process for warding off bacteria, wound healing, and for many critical processes for survival. Recent research has revealed that too much inflammation in some circumstances can have adverse effects, predominantly on the blood vessels that carry oxygen and nutrients throughout the body.[5] Atherosclerosis (the process that leads to cholesterol accumulation in the arteries) is essentially an inflammatory disorder of the blood vessels, just as arthritis is an inflammatory disorder of the bones and joints. Studies have also revealed blood markers that reflect the inflammatory process are elevated among individuals at high risk for future heart disease. Until recently, available markers of inflammation were not suitable for use in physicians' offices but CRP is very stable and easy to measure.

1.6 Interleukin 6

Interleukin 6 (IL-6) is a protein encoded by the IL-6 gene that acts as both a pro-inflammatory cytokine and as an anti-inflammatory myokine. Macrophages and T cells

stimulate immune response to trauma, infection, or other tissue damage that leads to inflammation and the secretion of IL-6. IL-6 is capable of crossing the blood-brain barrier and beginning synthesis of prostaglandin E2 in the hypothalamus to increase the body's temperature. IL-6 may also be secreted by macrophages in response to pathogen associated molecular patterns. These patterns bind to pattern recognition receptors in the innate immune system to induce intracellular signaling cascades that result in inflammatory cytokine production.

As a myokine, or a cytokine produced from muscle, IL-6 levels are elevated in response to muscle contraction. Therefore, it is significantly elevated with exercise and appears before other cytokines in the circulation. IL-6 signals through a type 1 cytokine receptor complex composed of the IL-6R alpha chain and the signal-transducing gp130 component.

The secretion of IL-6 is regulated by several physiologic and pathologic factors: hormones, cytokines, diet, physical activity, stress, hypoxia, and others. IL-6 plays a role in stimulating autoimmune and inflammatory processes in diseases such as depression, atherosclerosis, arthritis, diabetes, and some cancers. It is produced by many body cells such as adipose and liver cells but it is important to note that adipose tissue accounts for around 30% of IL-6 production. IL-6 release is related to adipose cells size and resultantly increased in obesity. Elevated levels of IL-6 also induce insulin resistance in liver and adipose cells by reducing the expression of insulin resistance substrate-1 (IRS-1)[6]. IRS-1 transmits the signal from insulin and insulin-like growth factor receptors to the PI3K/Akt and Erk Map kinase pathways to ultimately store glucose. Additionally, glucose transporting molecules that are regulated by insulin (GLUT-4) are also reduced

in adipose cells, further extending both the signaling and effector sections of insulin resistance.

Combined with the information that obesity has been shown to increase IL-6 and CRP serum levels and influenza vaccination is poorer in obese individuals, we wanted to investigate if whether these levels were related to BMI, age, and vaccine response in obese African-American females. African-American subjects were used in this study because they are an understudied population. Lack of adequate representation of African Americans in clinical cohorts continues to be the limiting factor in data ascertainment.

CHAPTER 2: SPECIFIC AIM AND HYPOTHESIS

Specific Aim: To measure the levels of IL-6 and CRP in obese African-American females and look for correlations between said levels and BMI, age, and vaccine response.

Hypothesis: There will be increased serum levels of IL-6 and CRP in subjects with higher BMI values, resulting in reduced vaccination response

CHAPTER 3: METHODS

3.1 Study Design

The subjects in this study were chosen from participants in an ongoing and prospective observational study at the University of North Carolina Family Medicine Center in Chapel Hill, NC. The parent study enrolled participants at least 18 years of age who were scheduled to receive the 2010-2011 inactive, trivalent seasonal flu vaccine. Participants were excluded if they had the following criteria: pregnant or breastfeeding women, self-reported use of immunomodulator or immunosuppressive drugs in the last 4 weeks, acute febrile illness, diseases such as HIV, hepatitis C, and cancer, and a history of hypersensitivity to any influenza vaccine components,

Informed consent, height, weight and baseline blood samples were collected from each participant at enrollment. They were then injected with one dose of the 2010-2011 trivalent inactive (0.5 mL Fluzone (Sanofi Pasteur, Swiftwater, PA, USA) A/California/7/2009 (H1N1)-like, A/Perth/16/2009 (H3N2)-like, and B/Brisbane/60/2008 (Victoria lineage)-like antigens) directly into their deltoid muscle. Participants returned to the UNC Family Medical Center 25-35 days after administration of the vaccine to collect a post-vaccination blood sample. Serum and peripheral blood mononuclear cells were isolated in both blood samples after collection. For this study, 19 total serum samples were used from African American women that did not smoke and that did not have diabetes. Ages ranged from 20 to 77 years old and BMIs fell between 30 and 61.4. Hemagglutination inhibition (HAI) titers were also used in this study, with samples being identified as having low (240≤) or high (240≥) HAI Perth (H3N2) antigen values.

3.2 Serum Collection

Serum was collected before receiving the vaccine and then again 25-30 days after receiving the vaccine. Pre- and post-vaccine blood draws were collected at the UNC Family Medicine Center in Chapel Hill, NC. Blood samples were collected into a vacutainer tube and then allowed to clot at room temperature for 30-60 minutes before being refrigerated at 4°C. The blood samples were then centrifuged at 800 x g for 10 minutes at 4°C using the IEC Centra MP4R. Following the centrifugation, 0.5 mL of the serum was aliquoted into 1.2 mL serum tubes and then stored in a freezer at a temperature of -80°C.

3.3 Hemagglutination Inhibition Assay

The hemagglutination inhibition assay is used to measure flu-specific antibody levels in blood serum. Serum antibodies to the hemmagglutinin surface glycoprotein (HA) interfere with the virus's ability to attach to red blood cells in this assay. Hemagglutination of the red blood cells is inhibited when antibodies are present at a sufficient concentration to bind the virus and prevent attachment to the red blood cells. Serial dilutions of serum are added to a known concentration of virus and red blood cells. The HAI titer is the highest dilution of serum that prevents hemagglutination. If the serum contains no antibodies that recognize the influenza strain HA, then red blood cell hemagglutination will be observed in all serum dilutions.

The HAI process began by treating the serum to be tested with receptor-destroying enzyme (RDE). RDE is used in the HAI test for the influenza virus in order to eliminate non-specific hemagglutination inhibitors existing in a serum specimen. RDE was added to each sample in a 1:3 ratio and then incubated at 37°C overnight. The

samples were cooled after being heat inactivated at 56°C for 1 hour and then 6 parts of physiological saline were added. The samples were frozen for 4 hours before use. The influenza stock virus was diluted to 8 HAU/50 ul and turkey RBCs were diluted to a concentration of 1% solution. Serial dilutions of test serum were added in duplicate to the wells of 96-well round bottom plates. Virus and RBCs were added to each well. Positive controls, negative controlT, and back titrations of the virus (to confirm virus concentration prior to performing the HAI) were also added to each plate. Hemagglutination was then read after 30 minutes at room temperature. If serum antibody is present, hemagglutination of the RBC will be inhibited. The HAI titer is the reciprocal of the last dilution of serum that completely inhibits hemagglutination.

3.4 Enzyme-Linked Immunosorbent Assay (ELISA) for Interleukin 6 (IL-6) and C-reactive protein (CRP)

The enzyme-linked immunosorbent assay (ELISA) was used to identify the levels of influenza-specific cytokines and proteins within the serum. The pro-inflammatory cytokine IL-6 and pentameric protein CRP levels were identified in the participants of this study. This technique enabled analysis of IL-6 and CRP levels by immobilizing them in microplate wells using antigen-antibody binding. The methods employed by these ELISAs were quantitative sandwich, or indirect, enzyme immunoassay techniques. A monoclonal antibody specific for CRP or IL-6 was pre-coated onto a microplate by the manufacturer. Standards and samples were pipetted into the wells and the immobilized antibody bound any CRP or IL-6 present. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for CRP or IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution

was added to the wells and color developed in proportion to the amount of CRP or IL-6 bound in the primary step. The color development was then stopped and the intensity of the color was measured by measuring the optical density using a microplate reader to determine the amounts of CRP and IL-6. A standard curve was created for each experiment and then the results were compared to each respective standard curve. The standard curve was created by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, and then drawing curve of best fit through the points on the graph. This color change signal was measured with an ELISA plate reader by measuring the absorbance at 450 nm. Increased enzyme activity (detected by a darker color reaction and higher optical density reading) was directly correlated with CRP or IL-6 concentration.

The ELISA procedures took one full day and were run concurrently. For the CRP assay, 100 µL of Assay Diluent and 50 µL of the standard or sample were added before being incubated for 2 hours. Each well was then aspirated and washed four times before adding 200 µL of CRP conjugate to each well and incubating for 2 hours at room temperature. The wash process was then repeated and 200 µL of substrate solution was added before incubated for 30 minutes at room temperature. 50 µL of stop solution were added and then the optical density of each well was recorded using a microplate reader. For the IL-6 assay, all the steps were similar except 100 µL of the standard or sample were added before the initial incubation. Additionally, the last incubation step after adding the substrate solution was 20 minutes rather than 30 minutes.

*Specific Details for Analysis CRP and IL-6 levels in Serum using ELISA***:**

Materials

Clear 96 well plates

Plate covers/sealers

Multi-channel precision pipettors with disposable plastic tips

Plate Reader

Reagents

CRP Conjugate	21 mL of monoclonal antibody against CRP conjugated to horseradish peroxidase with preservatives
CRP Standard	50 ng of recombinant human CRP in a buffered protein base with preservatives
Assay Diluent RD1F	6 mL of a buffered protein base with preservatives
Calibrator Diluent RD1F	21 mL of a buffered protein base with preservatives
Wash Buffer Concentrate	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives
Color Reagent A	12 mL of stabilized hydrogen peroxide
Color Reagent B	12 mL of stabilized chromogen
Stop Solution	6 mL of 2 N sulfuric acid
Human Serum	Serum samples of participants

Table 1. Reagents used during CRP ELISA.

IL-6 Conjugate	21 mL of polyclonal antibody specific for human IL-6 conjugated to horseradish peroxidase with preservatives
IL-6 Standard	1.5 ng of recombinant human IL-6 in a buffered protein base with preservatives then lyophilized
Assay Diluent RD1W	11 mL of a buffered protein base with preservatives
Calibrator Diluent RD5T	21 mL of a buffered protein base with preservatives
Calibrator Diluent RD6F	21 mL of animal serum with preservatives
Wash Buffer Concentrate	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives
Color Reagent A	12 mL of stabilized hydrogen peroxide
Color Reagent B	12 mL of stabilized chromogen
Stop Solution	6 mL of 2 N sulfuric acid

Human Serum	Serum samples of participants

Table 2. Reagents used during IL-6 Elisa

3.5 Statistical Analysis

The ELISA Data was analyzed using GraphPad Prism® software. Relationships between different factors (i.e. BMI, age, HAI concentration) were assed using the ANOVA test feature of the program. P-values <0.05 were deemed statistically significant.

CHAPTER 4: RESULTS

4.1 Demographics of the Study Population

The 17 participants were obese African-American females who were non-diabetic, did not smoke, and had a BMI over 30. African-Americans were the focus of this study because they are an understudied population. Participants of this study were broken down into two different categories on three occasions based on their BMI, age, and response rates to HAI titers of the H3N2 Perth virus. Low obese BMI was defined as ≤35 and high obese BMI as >35. Low age was defined as ≤50 and high age as >50. A low response to the H3N2 titer was defined as ≤240 and high response was defined as >240.

Participant-ID	Age	BMI	Gender	Race	Diabetes	Smoking
03-602-02	59	30.4	Female	aa	no	No
03-971-02	42	35.0	Female	aa	no	No
03-681-02	46	38.8	Female	aa	no	No
03-738-02	42	52.4	Female	aa	no	No
03-783-02	26	36.6	Female	aa	no	No
03-817-02	64	31.8	Female	aa	no	No
03-934-02	53	33.8	Female	aa	no	No
03-633-02	54	38.6	Female	aa	no	No
03-815-02	57	30.3	Female	aa	no	No
03-978-02	54	34.1	Female	aa	no	No
03-939-02	77	31.5	Female	aa	no	No
03-948-02	23	54.7	Female	aa	no	No
03-733-02	48	38.5	Female	aa	no	No
03-714-02	51	41.1	Female	aa	no	No
03-804-02	63	36.1	Female	aa	no	No
03-552-02	37	61.4	Female	aa	no	No
03-671-02	20	36.5	Female	aa	no	No

Table 3. Demographics of the participants in this study. All the participants were African-American women with BMIs between 30.4 and 61.4 kg/m^2 . The average age of the participants was 48 and the average BMI was 38.9.

	High Age Group	Low Age Group	Low BMI Group	High BMI Group	Low HAI Group	High HAI Group
Mean	59.1	35.5	32.4	43.5	85.7	624

Table 4. Means of the selected groups and variables tested

4.2 Cytokine Levels in Serum (IL-6 and CRP)

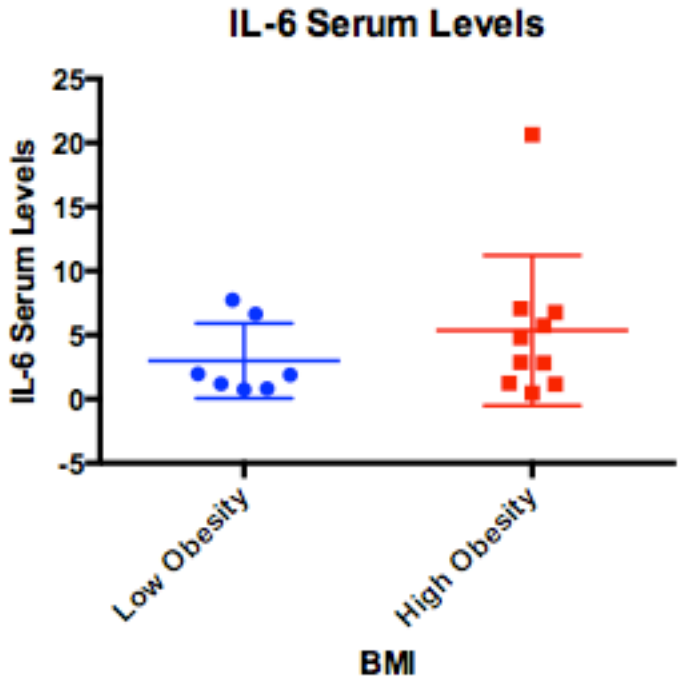
Participant-ID	Age	BMI	IL6 Concentration (pg/mL)	CRP Concentration (mg/L)	HAI of Perth (H3N2)
03-602-02	59	30.4	0.823	0.184	0
03-971-02	42	35.0	0.727	0.819	0
03-681-02	46	38.8	1.169	1.61	80
03-738-02	42	52.4	20.629	1.599	80
03-783-02	26	36.6	6.766	1.925	120
03-817-02	64	31.8	1.892	1.243	160
03-934-02	53	33.8	1.193	0.284	160
03-633-02	54	38.6	1.241	0.651	320
03-815-02	57	30.3	7.749	0.225	320
03-978-02	54	34.1	1.954	0.894	320
03-939-02	77	31.5	6.634	2.171	320
03-948-02	23	54.7	7.05	2.094	320
03-733-02	48	38.5	2.812	0.444	480
03-714-02	51	41.1	4.798	1.976	640
03-804-02	63	36.1	0.469	2.135	960
03-552-02	37	61.4	5.731	2.221	1280
03-671-02	20	36.5	2.857	1.533	1280

Table 5. Average CRP and IL-6 levels in the serum of participants. All results were derived from the ELISA plate reader.

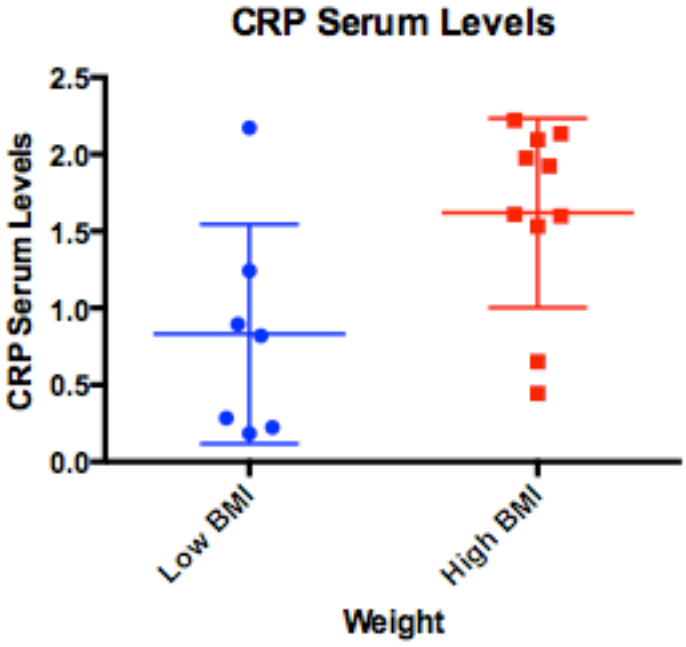
Mean	IL6 (pg/mL)	CRP (mg/L)	HAI for H3N2
Low BMI	4.742714286	1.094857	85.714
High BMI	4.1295	1.4344	624

Table 6. Average IL6 and CRP values for the low and high BMI groups.

The data was taken from the plate reader and then analyzed using Prism's ANOVA feature to find any correlations between serum levels of IL-6 and CRP and BMI value.

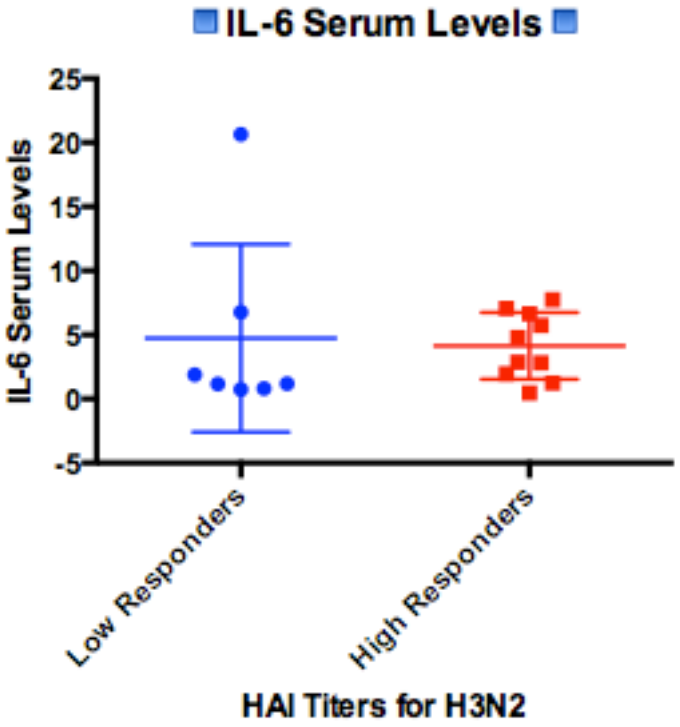


Graph 1. The IL-6 serum levels (in pg/mL) for the low obesity and high obesity groups were 2.996 ± 1.105, n=7 and 5.352 ± 1.855, n=10, respectively. There was no significant difference to conclude a link between BMI and IL-6 serum levels, as the p-value was equal to 0.1040.

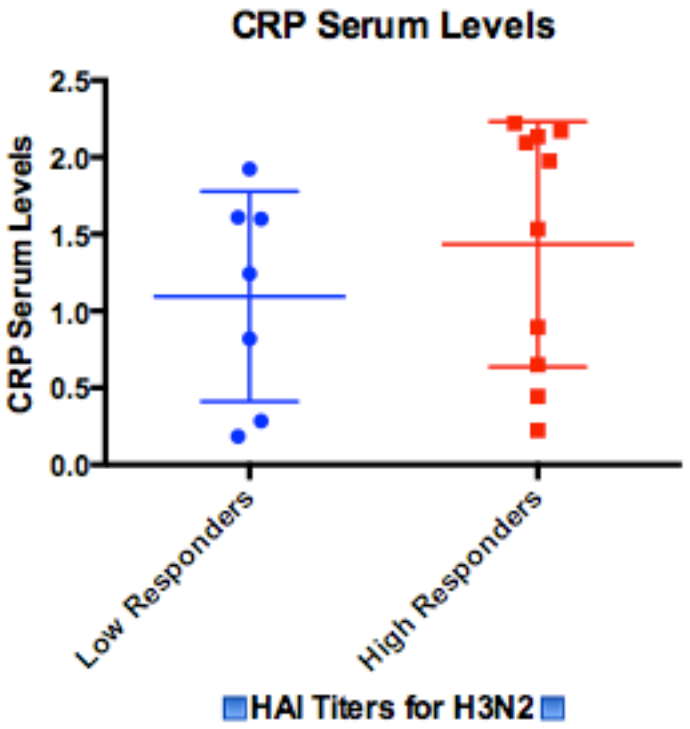


Graph 2. The CRP serum levels (in mg/L) for the low obesity and high obesity groups were 0.8314 ± 0.2697, n=7 and 1.619 ± 0.1944, n=10, respectively. There was no significant difference to conclude a link between BMI and CRP serum levels., as the p-value was 0.6598.

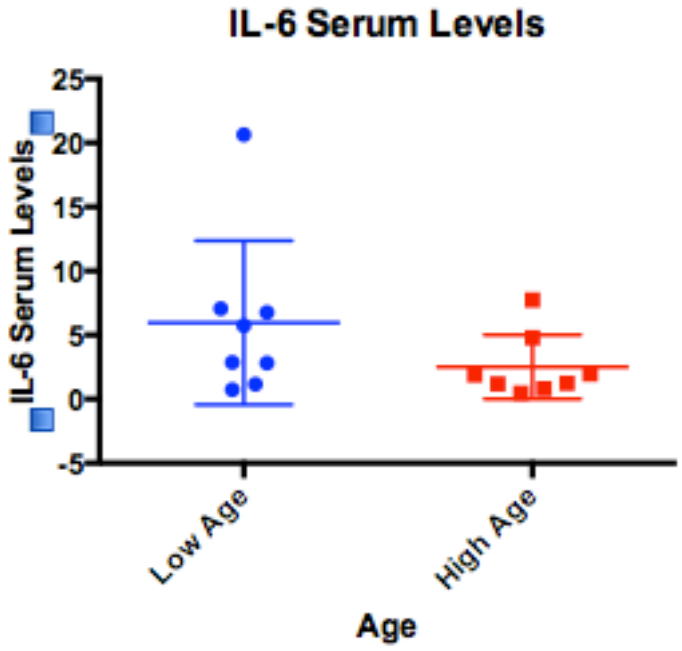
There seems to be a trend in this data towards higher levels of CRP in the high BMI group. Statistical significance is not reached though because of a limited number of subjects.



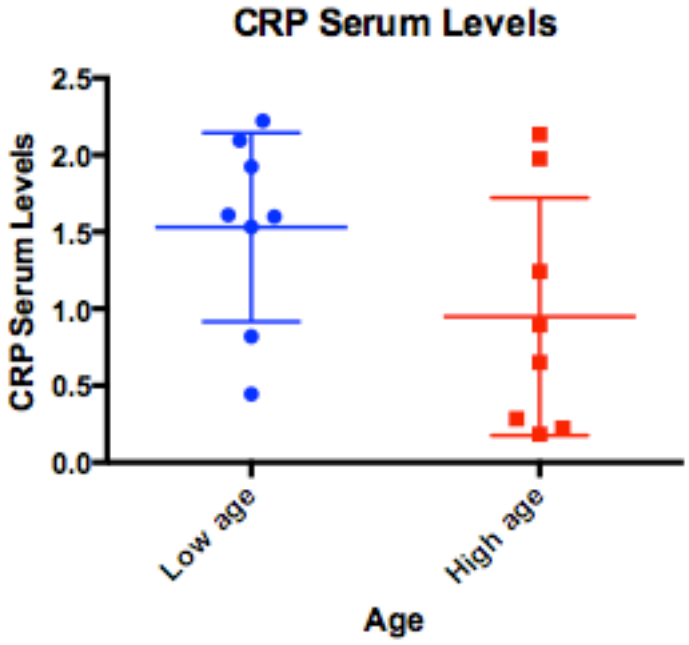
Graph 3. The IL-6 serum levels (in pg/mL) for the low responding and high responding groups were 4.743 ± 2.767, n=7 and 4.130 ± 0.8215, n=10, respectively. There was a significant difference to conclude a link between response to HAI titers for H3N2 and IL-6 serum levels, as the p-value was 0.0069.



Graph 4. The CRP serum levels (in mg/L) for the low responding and high responding groups were 1.095 ± 0.2579, n=7 and 1.434 ± 0.2525, n=10, respectively. There was no significant difference to conclude a link between response to HAI titers for H3N2 and CRP serum levels, as the p-value was 0.7245.



Graph 5. The IL-6 serum levels (in pg/mL) for the low and high age groups were 5.968 ± 2.264, n=8and 2.515 ± 0.8837, n=8, respectively. There was a significant difference to conclude a link between age and IL-6 serum levels, as the p-value was 0.0239.



Graph 6. The CRP serum levels (in mg/L) for the low and high age groups were 1.531 ± 0.2174, n=8 and 0.9490 ± 0.2736, n=8, respectively. There was no significant difference to conclude a link between age and CRP serum levels., as the p-value was 0.5593.

There seems to be a trend in this data towards lower levels of CRP in the high age group. Statistical significance is not reached though because of a limited number of subjects.

CHAPTER 5: DISCUSSION

5.1 Conclusions

A subset of obese, non-diabetic, non-smoking African American women were investigated in this study to better understand factors that contribute to the variation in responses to the influenza vaccination. The original hypothesis stated that increased levels of IL-6 and CRP in subjects with a high BMI would be correlated with a lower response to vaccination. Obese individuals typically have higher levels of inflammation, and consequently, higher levels of IL-6 and CRP. Adipocytes serve as not only a means of storage for body energy but also as endocrine organs with metabolic roles that regulate the body's physiology. Enlarged adipocytes in obese individuals promote inflammation through the recruitment of macrophages and the release of factors that also prompt insulin resistance.[15] This study confirmed that obese individuals had higher concentrations of IL-6 and CRP compared to healthy weight individuals. It is important to note though that increasing obesity did not lead to an increase of in inflammatory markers within this study. This lack of significance may be attributed to the limited and non-diverse sample size, with respect to varying obesity.

Overall, no significant link was found between increasing obesity and response to the influenza vaccine. Once an individual reaches obesity, or a BMI over 30, the levels of inflammation rates more or less taper off and become stagnant. Increases in obesity have not been shown to increase inflammation at the rate at which an individual approaching obesity would. Both IL-6 and CRP serum levels were deemed independent of body mass index. Both IL-6 and CRP serum levels were higher in these obese subjects than the normal levels for healthy weight people, which average below 1.0 mg/L.[16] The low BMI

group had an average level of 1.09 mg/L and the high BMI group had an average level of 1.43, further signifying that increased weight increases CRP levels. Increased body weight results in higher rates of inflammation so these results make sense. Normal IL-6 levels are between 1-2 ug/mL.[17] The lower BMI group had an average of 4.74 ug/mL of IL-6 in their serum while the high BMI group had an average of 4.12 ug/mL. These results are consistent with obese individuals having higher serum levels. The fact that the lower weight group had larger values than the higher way group may be a result of a rather limited sample size.

The results from this experiment confirmed that there was statistically significant data to show a link between IL-6 serum levels and a response the HAI titer for the H3N2 virus. The higher response group had lower IL-6 serum levels and the lower response group had high IL-6 serum levels. IL-6 is a cytokine that serves a variety of immune functions in response to acute illness or injury. As a pro-inflammatory cytokine, IL-6 is involved in activating inflammatory pathways. If IL-6 levels are low, then the body does not require any inflammation and is in a generally healthy state. Additionally, a significant difference was found to support a link between IL-6 serum levels and age. Younger subjects had higher IL-6 serum levels than older subjects.

CRP is produced by the liver and rises when there is inflammation throughout the body. Its purpose is to activate the complement system, which helps antibodies clear pathogens from organisms, by attaching to dead cells after IL-6 is released. Surprisingly this particular study established links between age, HAI response, or BMI for CRP serum levels. These unforseen results may have been attributable to the small sample size.

5.2 Limitations and Recommendation for Further Studies

This study had multiple limitations that may have skewed or produced inaccurate data. The CRP and IL-6 ELISA kits were both expired at the time of the experiment. This may have resulted in degraded standard and stopping solutions, and as a result produced inaccurate data outcomes for the subjects. Additionally the kits mays have lost a significant amount of sensitivity in the process. A degraded standard would have compared sample values to an incorrect value while a degraded stopping solution may have lost its effectiveness in stopping the reaction after the substrate was added because of a decreased concentration. This ineffective stopping reagent would have resulted in overvalued concentrations of IL-6 and CRP in the samples. This limitation could be avoided in future experiments by procuring new ELISA kids ahead of time to ensure all products and solutions create accurate data.

Another limitation of the experiment is that procedural mistakes may have occurred in the ELISA process. Four ELISA experiments were conducted concurrently, increasing the potential risk of error. Meticulous following of protocol is essential to ensuring accurate results. The subject samples were dispersed throughout the lab and had to be acquired in a timely manner before beginning the experiment. This resulted in the samples being thawed and refrozen on multiple occasions before being processed in the ELISA. The freezing and thawing process affects the levels of cytokines in the plasma. In future experiments, it is imperative that ELISA experiments are run immediately after the serum is collected.

A major limitation in this experiment is that there was a small sample size of only 17 subjects. There were only 17 obese African-American females available in the data set who did not have diabetes and who did not smoke. These subjects were chosen to limit

any differences in between subjects and get an accurate reading of just cytokine levels. Having a small data set reduced the statistical power of the study, making it difficult to account for the large variability in the results. Additionally, no lifestyle confounders or variables were considered. Subjects were not asked about their physical activity levels, eating habits, or about any other health conditions that could have affected cytokine levels. In future experiments, a larger sample size should be used and lifestyle factors should be considered. Furthermore, studying a non-obese group would be helpful as well to better understand the effects of BMI on cytokine levels.

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